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bryA: An Unusual Modular Polyketide Synthase Gene from the Uncultivated Bacterial Symbiont of the Marine Bryozoan *Bugula neritina*

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Summary

"*Candidatus* Endobugula sertula," the uncultivated bacterial symbiont of *Bugula neritina*, is the proposed source of the bryostatin family of anticancer compounds. We cloned a large modular polyketide synthase (PKS) gene complex from "*Candidatus* Endobugula sertula" and characterized one gene, *bryA*, which we propose is responsible for the initial steps of bryostatin biosynthesis. Typical PKS domains are present. However, acyltransferase domains are lacking in *bryA*, and β-ketoacyl synthase domains of *bryA* cluster with those of PKSs with discrete, rather than integral, acyltransferases. We propose a model for biosynthesis of the bryostatin D-lactate starter unit by the *bryA* loading module, utilizing atypical domains homologous to FkbH, KR, and DH. The *bryA* gene product is proposed to synthesize a portion of the pharmacologically active part of bryostatin and may be useful in semisynthesis of clinically useful bryostatin analogs.

Introduction

Marine invertebrates, particularly sessile ones, are rich sources of unusual metabolites. Like terrestrial plants, they often rely upon chemical defense to discourage predation. Symbiosis with biochemically versatile microorganisms is an efficient strategy to accomplish such chemical defense. Microbial symbionts have often been invoked as biosynthetic sources of natural products found in marine invertebrates, based on structural similarities to known microbial compounds. These compounds may have applications in a clinical setting, but their development is often difficult due to scarcity of supply, and solutions to this problem can be elusive. In some cases, chemical synthesis is impractical, and not all invertebrates are amenable to aquaculture. Finally, cultivation of symbionts suspected as biosynthetic sources of bioactive compounds from invertebrates is often difficult. However, symbiotic systems are accessible to investigation of biosynthesis by using molecular biological techniques [1]. Because genes in bacterial pathways are often contiguous in the genome, biosynthetic genes can be cloned directly from the symbionts and can be reconstituted in a heterologous host for expression and, ultimately, compound production. Once the genes are in hand, combinatorial or pathway-engineering approaches can be used to obtain novel structures with different activities. An example of this approach is the isolation of genes proposed to code for the biosynthesis of the bioactive polyketide, pederin, from a bacterial symbiont of the blister beetle, *Paederus fuscipes* [2].

Bryostatins are a family of cytotoxic macrolides found in the marine bryozoan, *Bugula neritina* (Figure 1). They are chemically interesting because of their unique structures, large size, and biological activity. Bryostatins act as a chemical defense for *B. neritina* larvae by making them unpalatable to fish predators [3, 4]. In addition to this role, bryostatins have anticancer activity, and bryostatin 1 is in clinical trials in combination with other chemotherapeutic agents for treatment of a variety of cancers (http://www.clinicaltrials.gov) [5–16]. Several decades have elapsed between the discovery of the bryostatins and their clinical application because of difficulties in obtaining a supply of these scarce compounds.

Bryostatins resemble bacterial complex polyketides, suggesting that they are likely to be biosynthesized by a modular polyketide synthase (PKS) mechanism. Modular PKSs are protein complexes that synthesize polyketide structures by a stepwise chain elongation mechanism and use acyl-CoA substrates as precursors. The growing chain is attached to the complex by a β-ketothioester linkage. Each step in chain extension and modification is carried out by modules consisting of core and accessory domains with different catalytic activities [17, 18]. Core domains consist of a β-ketoacyl synthase (KS) and an acyl carrier protein (ACP), which are responsible for condensing successive acyl-CoA substrates that are selected and activated by an acyltransferase (AT). In an individual module, the enzyme bound β-ketothioester can be reduced at the keto group to varying degrees by accessory domains. These include, in the order in which they are used, the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains [17, 18]. After completion of the steps in one module, the chain is passed on to the next. Because modules function independently, modular PKSs can evolve to create tremendous molecular diversity. Some of this diversity stems from the possibility of variations to the conventional PKS scheme. In some PKS gene clusters, specific domains can be present but function-

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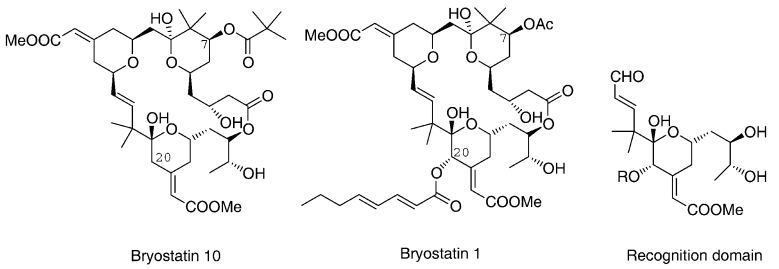

Figure 1. The Structure and Recognition Domain of the Bryostatins

Structure of bryostatins and the recognition domain of bryostatin [29, 53]. Bryostatin 10 has been shown to be a feeding deterrent in *B. neritina* larvae [4] and probably is the ecologically significant compound. Bryostatin 1 is currently in clinical trials for a variety of cancer treatments. The recognition domain is the part of the bryostatin molecule essential for its anticancer activity [29, 53].

ally inactive [19], whereas, in others, a single domain or entire module can function in more than one round of elongation [20]. Yet, other clusters exhibit module skipping, wherein the growing polyketide chain can skip over an entire module, allowing another level of molecular diversity [19].

B. neritina has recently been shown to be a complex of at least three sibling species that vary in the types of bryostatins they produce [21, 22]. There are 20 described bryostatins [4, 23–26]; most of their diversity is due to variations at the C-7 and C-20 positions (Figure 1). One sibling species, type D (deep), produces bryostatin 1 and other bryostatins with a 2,4-octadienoate substituent at C-20 [22], whereas the other species, type S (shallow), produces bryostatins without this addition at C-20 (Figure 1). The bryostatins of the third sibling species, Northern Atlantic, have not been characterized. Because the predominant bryostatins in the deep and shallow species differ only in the C-20 substituent, we expect that a common precursor of the bryostatins is synthesized by a similar modular polyketide synthase in both. *B. neritina* contains a bacterial symbiont, "*Candidatus* Endobugula sertula" (abbreviated "*Candidatus* E. sertula"), which is believed to be responsible for synthesizing the polyketide precursor to the bryostatins [27]. Reduction or elimination of "*Candidatus* E. sertula" from *B. neritina* colonies by antibiotic treatment of larvae reduces bryostatin production in the new colony and eliminates it in the next generation of larvae with no effect on colony growth. This finding suggests that the symbiont does not play a major nutritional role [4, 27]. The yield of bryostatins from *B. neritina* is low, and thus far it has not been possible to cultivate "*Candidatus* E. sertula," limiting the supply of bryostatin. Chemical synthesis of bryostatins is possible, but the complexity of the procedure makes it impractical as a means to provide a clinical supply [28]. A promising avenue is synthesis of simpler analogs of bryostatins based on the pharmacologically active portion of the molecule (Figure 1), or "recognition domain" [29]. Another approach for obtaining part of or the entire bryopyran ring of bryostatins, which is likely to be very cost effective, would be to clone the bryostatin synthesis genes and express them in a heterologous host. In previous work, we isolated and cloned a 300 bp β-ketoacyl synthase gene fragment (KSa) from a total DNA preparation from *B. neritina* by PCR with degenerate primers [27]. The KSa gene fragment was present in all *B. neritina* populations tested, but not in other bryozoans, and other KSs obtained were only sporadically present and were proposed to originate from casual bacterial associations. Using in situ hybridizations, it was determined that KSa gene fragment transcripts were located and expressed in "*Candidatus* E. sertula" cells in *B. neritina* larvae [27]. Furthermore, the KSa gene fragment declined in abundance after antibiotic treatment that specifically reduced the "*Candidatus* E. sertula" population in *B. neritina* colonies [27]. These results provided evidence that KSa is encoded and expressed in "*Candidatus* E. sertula." We hypothesized that KSa is part of a larger modular polyketide synthase-encoding complex responsible for biosynthesis of the bryopyran precursor to bryostatins, and that KSa could be used as a probe to isolate the entire gene cluster. This report describes the use of such an approach to clone the first gene of a PKS cluster that we believe is responsible for bryostatin synthesis.

Results

Enrichment of "*Candidatus* E. sertula" DNA Content in Total DNA

Total DNA extracted from *B. neritina* consists of a mixture of DNA from the bryozoan, epibionts, "*Candidatus* E. sertula," and potentially DNA of other associated bacteria. Consistent with this, examination of the bacterial community associated with the colony by amplification of bacterial small ribosomal subunit (16S) genes, followed by denaturing gradient gel electrophoresis, revealed a complex community [27]. In order to facilitate library construction and Southern blotting experiments, we developed procedures to enrich the "*Candidatus* E. sertula" content of DNA extracts. We used competitive PCR to quantify the enrichment at different stages of purification, with a cloned competitor fragment derived from the previously isolated KSa gene fragment [27]. These assays indicated a 5.5-fold enrichment using differential centrifugation to enrich for bacteria, and a total of 16-fold enrichment in particular fractions isolated from subsequent Hoechst dye density gradients (see figure 6 in reference [1]).

Cloning *bryA*

It was determined that only a total DNA preparation of *B. neritina* from Mission Bay (not "*Candidatus* E. sertula"-enriched) provided DNA of sufficient size for cosmid cloning. A cosmid library was constructed by using total DNA from Mission Bay *B. neritina* and was screened with the KSa gene fragment. Four positive cosmids with inserts of 30–40 kbp were isolated and sequenced from the ends and from internal restriction sites. These cosmids spanned a region of PKS homology of approximately 65 kbp (the *bry* cluster). At this stringency, the

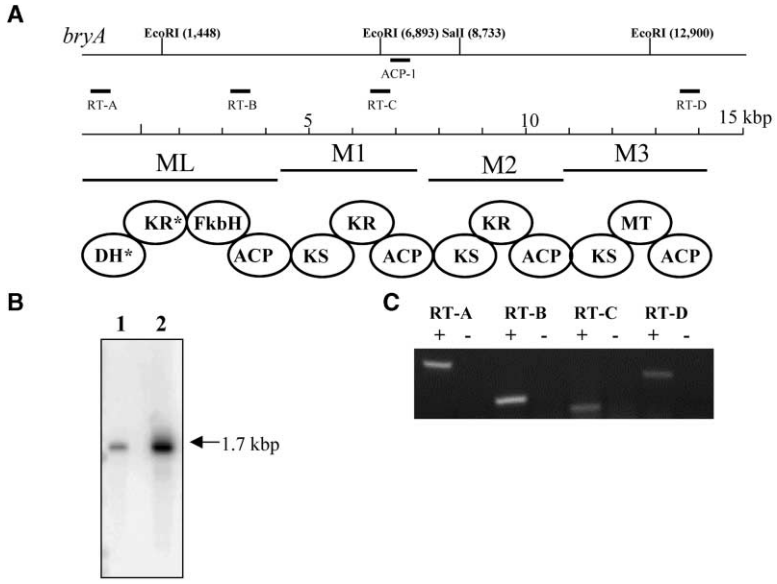

Figure 2. Genetic Analysis of the *bryA* Region

(A) Map of *bryA* indicating locations of probes, reverse transcription (RT)-PCR products, and module and domain content. The top portion shows a restriction map; ACP-1 indicates the location of the probe used in the Southern blot in (B). Below the map are locations of RT-PCR products examined in (C), and under these is a nucleotide scale bar in kilobase pairs (kbp). Below the scale bar are delineated the locations of the four modules in the *bryA* gene, and below this are the relative arrangements of domains—these are not precisely to scale. Domain abbreviations are as follows: DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein; KS, β-ketoacyl synthase; MT, methyltransferase; FkbH, FkbH homolog originally identified in the ansamitocin biosynthetic cluster [31]. (B) Southern blot hybridization of EcoRI/SalI-digested genomic "*Candidatus* E. sertula"-enriched DNA (lane 1) and cloned DNA (lane 2); radiolabeled ACP-1 is used as a probe. (C) Agarose gel of *bryA* RT-PCR products derived from adult Scripps Pier *B. neritina* RNA showing the presence of transcripts throughout the *bryA* ORF. Regions amplified are denoted at the top (RT-A through D), and lanes in which the protocol included (+) or omitted (−) the reverse transcriptase are shown.

KSa probe identified cosmids spanning the entire 65 kbp region, not just those in which KSa was located. No clones were isolated that did not map to the *bry* cluster. Two of the cosmids showed evidence of deletions upon propagation. To minimize such artifacts, a second library was prepared by using "*Candidatus* E. sertula"-enriched DNA of the shallow genotype from Scripps pier, which was cloned into Lambda Dash and yielded smaller inserts from 10 to 20 kbp.

This library was screened repeatedly at low stringency with probes spanning 91% of the 65 kbp *bry* cluster. A total of 53 positive clones were identified and end sequenced, and 32 clones mapped to the *bry* cluster. Six were cloning artifacts, in which *bry* cluster Sau3A fragments were ligated to Sau3A DNA fragments from elsewhere in the genome. These, plus the 32, represent 75% of the positive clones. Eleven clones did not contain PKS homology on either end. These represent either small clusters (<15 kbp) or cloning artifacts. Four clones contained PKS sequence not found in the *bry* cluster. It is unknown whether these genes are linked, but if they are, the cluster in which they originate would be small. If these four come from a single cluster, its representation (7.5% of total positives) suggests that it is from a cluster approximately one-tenth the size of the *bry* cluster. Although the low representation of these clones could be attributed to cloning bias, low-stringency Southern analysis, which is not subject to this bias, corroborates these data (see "Genomic Mapping" below). The fact that non-*bry* PKS clones were isolated demonstrates that the stringency used was low enough to detect other clusters. These results strongly suggest that the *bry* cluster is the only PKS in the "*Candidatus* E. sertula" genome large enough to be responsible for the biosynthesis of bryostatins.

Four overlapping lambda clones spanned the *bryA* gene, and this region was fully sequenced from these clones (Figure 2A). To compare the PKS region of the deep and shallow genotypes, a third library was prepared from "*Candidatus* E. sertula"-enriched DNA, extracted from the deep genotype from Torrey Pines Artificial Reef, cloned into Lambda ZAP (insert size about 5 kb), and screened with probes from the shallow genotype; no clones were identified that did not originate in the *bry* cluster. Three overlapping clones spanned the last two-thirds of the *bryA* gene and were fully sequenced. The upstream portion could not be isolated from the Lambda ZAP library due to the fact that the size of the target fragment was larger than that selected for cloning, resulting in underrepresentation in the library. Using sequence information from the shallow genotype, four overlapping PCR products were amplified directly from genomic DNA, which completed the deep *bryA* sequence.

Genomic Mapping

We verified that the sequence data obtained from the cloned *bryA* gene sequences corresponded to restriction maps of genomic DNA by using Southern blot hybridization with "*Candidatus* E. sertula"-enriched DNA. Four probes were designed that spanned the *bryA* region, and the DNA was digested with seven different combinations of restriction enzymes, of which only EcoRI and SalI sites proved to be present in the *bryA* region. Other enzymes that cut outside of the region confirmed the sizes of fragments at the ends of *bryA*. The map deduced from the sequence corresponded to the sites observed in the genomic DNA blots. A representative Southern blot is shown in Figure 3B, which identifies the same size gene fragment in genomic and cloned DNA. When probed at low stringency and examined with image enhancement, total DNA contained additional hybridizing bands not originating in the *bry* cluster. All but one of these are absent in "*Candidatus* E. sertula"-enriched DNA, indicating that they originate from environmental bacteria, not from the "*Candidatus*

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ACP          1163
bryA ACP-L NSNLENYGLDSYAIINIVVEL
bryA ACP-1 KRNLADFGFDSIRLKEFAHFL
bryA ACP-2 QAPLERYGIDSLIVIQVNQAL
bryA ACP-3 LKPFTDLGLDSINGVTWIRKI
eryA ACP-1 EATFRELGLDSVLAAQLRAKV
                    *

KS             1505             1640        1682
bryA     KS-1 DTACSSSGTAVHLA  VEAHGTGTPL  NIGHLES
bryA(S)  KS-2 NTACSSALVALHQA  IVAHGTGTQL  NFGHTFA
bryA(D)  KS-2 DTTCSSALVALHQA  IVAHGTGTQL  NFGHTFA
bryA     KS-3 DTMCSSSLTAIHEA  IEAHGTGTEL  NIGHLEA
eryA     KS-1 DTACSSSLVAVHLA  VEAHGTGTRL  NLGHTQA
                 *

KR           283              375
bryA KR*-L LLTGASRGLGAAFAR  GHLSERYIEQNLEL
bryA KR-1  LITGGAGKLGLLFAR  GVIHAAGIQHNQSI
bryA KR-2  LISGGVGGIGLHIAH  GIVHSAGITRDNFI
eryA KR-1  LVTGGTGGVGGQIAR  AVFHAAATLDDGTV
eryA KR-2  LVTGGTAGLGAEVAR  GVVHAAGLPQQVAI
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Figure 3. Amino Acid Alignments of Conserved Regions of the *bryA* PKS Domains

Conserved amino acid sites in the *bryA* PKS domains [54]. Only the regions containing proposed active sites are shown. The numbers correspond to the amino acid position of the first residue in the first motif listed. Active site residues are indicated by asterisks, and residues important for function are shown in bold. The KS-2 domains with significant amino acid differences from deep (D) and shallow (S) "*Candidatus* E. sertula" are annotated as such. The KR domain motif is underlined (GxG/AxxGxxxA). Also shown is the B-type stereochemistry signature (LDD) for *eryA* KR-1; *eryA* KR-2 has A-type stereochemistry. Domains from the erythromycin gene cluster (*eryA*) are shown for comparison.

E. sertula" genome (data not shown). The remaining band is not large enough to encode a bryostatin cluster, and it may correspond to a non-*bry* PKS isolated from the Lambda Dash "*Candidatus* E. sertula"-enriched DNA library.

Sequence Analysis

Sequence analysis indicated that *bryA* comprises a single open reading frame (ORF) of 14,664(deep)/14,667 (shallow) nucleotides, encoding 4,888(deep)/4,889(shallow) amino acids. The overall GC content of the gene is low at 38%. The four non-*bry* PKS fragments found through cloning also contain an overall low GC content of between 32% and 42%. Although it is possible that these fragments originate in other bacteria associated with "*Candidatus* E. sertula," this result suggests that they probably originate in "*Candidatus* E. sertula" but are part of one or more smaller PKS clusters. In the deep sequence, nucleotides 9466–9468 are missing, which results in the deletion of one amino acid. *bryA* genes from the shallow and deep sibling species were very similar, with 98.3% identity at the nucleotide level and 97.8% at the amino acid level. The major differences were found in one region between nucleotides 7753 and 8994, where the nucleotide identity was 90.3%, as opposed to >99% in the rest of the gene.

Domain Content

Using BLAST [30] on the NCBI server, we determined that the greatest overall similarity of the *bryA* genes was to the *Bacillus subtilis pksM* genes and the *Paederus fuscipes* symbiont *ped* genes. The *bryA* gene contains 13 identifiable domains organized into 4 modules (ML, M1–M3) (Figure 2A). Locations of the domains (based on amino acid position) in each module are listed in Table 1. All four modules contain an ACP. M1–M3 contain β-ketoacyl synthases, and M1 and M2 each contain a ketoreductase. M3 contains a methyltransferase (MT). The loading module (ML) is quite unusual. The first domain in this module is most similar to a dehydratase, and the second is most similar to a ketoreductase; however, neither are an exact match throughout their sequence. Finally, the loading module contains a homolog of FkbH, a gene first identified in the ansamitocin gene cluster [31]. Most domains of M1–M3 appear to contain the active site consensus sequences for function, including the region with major differences between deep and shallow, where the KS of M2 is located (Figure 3). The FkbH homolog in ML and a MT domain present in M3 are not common; however, similar domains have been observed in other PKS systems [31–34]. Phylogenetic analysis indicated that *bryA* KS domains clustered with KS domains from other modular Type I PKS genes that contain discrete acyltransferase (AT) domains, and not with those PKS genes that contain integral ATs (Figure 4). This is consistent with the lack of ATs in the *bryA* sequence and suggests that one or more discrete ATs are located elsewhere in the genome.

Table 1. Locations of Domains in the *bryA* Sequence

Module	Domain	Amino Acid Position
ML	DH*-L	1–124
	KR*-L	278–490
	FkbH-L	518–1026
	ACP-L	1131–1203
M1	KS-1	1338–1805
	KR-1	2207–2393
	ACP-1	2472–2543
M2	KS-2	2582–3031
	KR-2	3377–3571
	ACP-2	3651–3720
M3	KS-3	3778–4248
	MT-3	4473–4755
	ACP-3	4756–4829

Native RNA Expression

Due to the low content of "*Candidatus* E. sertula" RNA in total RNA preparations from *B. neritina*, reverse transcription PCR (RT-PCR) was used to determine if *bryA* was transcribed in vivo. RT-PCR demonstrated the presence of transcripts from regions A, B, C, and D of *bryA* (Figures 2A and 2C). The identity of the RT-PCR products was confirmed by sequencing. Control RT-PCRs performed without reverse transcriptase were negative (Figure 2C), verifying that DNA contamination did not contribute to the RT-PCR products produced with reverse transcriptase (Figure 2C). These results are consistent with transcription of the entire *bryA* ORF in *B. neritina*.

Discussion

The sequences of *bryA* from the deep and shallow species of *B. neritina* are remarkably similar. The structure

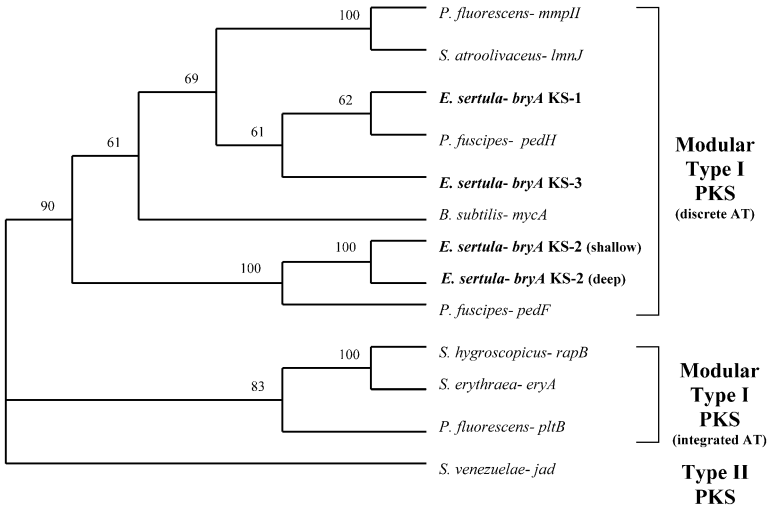

Figure 4. Phylogenetic Tree of KS Amino Acid Sequences Generated by Using Distance and Heuristic Search in PAUP

Bootstrap values for clades are displayed at the nodes. KS domains from *bryA* in "*Candidatus* E. sertula" (including the KS-2 domains from both deep and shallow species) and several modular Type I PKS genes are compared. A Type II PKS KS from *S. venezuelae* is used as the outgroup. Groupings indicate modular Type I PKS gene clusters that contain discrete AT domains (a single domain for entire cluster) or integrated AT domains (domains present reiteratively in all modules).

and domain organization of the genes from both species is identical, and amino acid identity is very high. Although one region is less conserved than the rest of the gene, all conserved residues recognized as important for domain function in one species are conserved in the other. The existence of such a large ORF in a symbiont genome, where genome reduction or corruption might be expected to occur [35], and the demonstration that the ORF is transcribed throughout its length, suggest that *bryA* is functional. Extensive library screening, Southern hybridizations, and sequencing revealed no evidence of large PKS clusters other than the *bry* cluster containing *bryA*, suggesting that other large modular PKSs are absent in "*Candidatus* E. sertula."

A hypothesis for the function of *bryA* in bryostatin biosynthesis is shown in Figure 5. We propose that the first module within *bryA* is a loading module, which consists of four domains, including a DH homolog (DH*), a KR homolog (KR*), FkbH, and ACP (Figure 5). FkbH homologs have recently been observed in other PKS gene clusters, and the function of FkbH has been attributed to catalyzing, in association with other protein components, the formation of methoxymalonate extender units from a glycolytic pathway intermediate [31, 34]. By analogy, using the same intermediate, a sequence of three reactions catalyzed by FkbH, DH*, and KR* will yield D-lactate (Figure 5A), the expected starter unit for bryostatin biosynthesis. In the first reaction, the glycolytic pathway intermediate, presumably phosphoglycerate, is transferred to ACP by the action of FkbH. A dehydration reaction catalyzed by DH* then occurs, generating an enzyme bound enoylpyruvate, which can easily rearrange to form an enzyme bound pyruvate. In the final reaction, the KR* reduces the α-keto group of pyruvate, and the system is ready for the first condensation-reduction cycle to be catalyzed by the downstream extension module in *bryA* (Figure 5B). This scheme is consistent with the observation by Kerr et al. [36] that radiolabeled glycerol was incorporated into bryostatin, presumably as a precursor of the 3-carbon starter unit. In bacteria, glycerol enters into glycolysis through phosphorylation and oxidation to dihydroxyacetone phosphate, which in turn is isomerized to glyceraldehyde 3-phosphate. The latter is then oxidized to form phosphoglycerate.

The α-ketoreduction we propose for the KR* is unusual in complex polyketide biosynthesis. Normally, a KR in a modular PKS system catalyzes reduction of

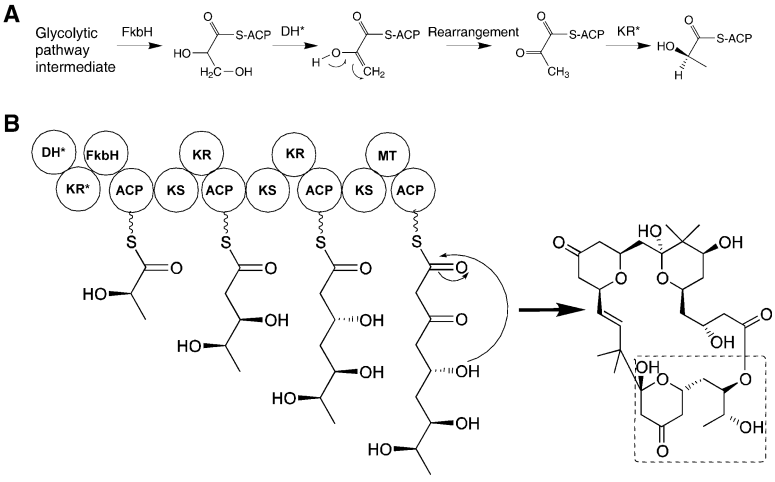

Figure 5. Proposed Involvement of BryA in Bryostatin Synthesis

(A) The proposed pathway for D-lactate formation by the bryostatin loading module.

(B) The proposed series of reactions catalyzed by BryA. BryA consists of the loading module and three chain extension modules. The curved arrow indicates the position of lactonization. The dashed box depicts the part of bryostatin that BryA synthesizes.

β-ketoacyl ACP generated during elongation of the polyketide chain. Most (if not all) KR domains, including the KR* in BryA, are most similar to a diverse family of enzymes collectively known as short-chain dehydrogenase/reductases (SDR). Enzymes included in the SDR family span several EC classes, with oxidoreductases forming the majority [37]. When we first speculated on the reaction scheme shown in Figure 5A, the only known groups of enzymes involving FkbH and presumably synthesizing a 3-carbon polyketide building unit from a glycolytic pathway intermediate were those reported for FK520 and ansamitocin biosynthetic gene clusters [31, 34]. However, A BLAST [30] search analysis of the *bryA* region revealed that the amino acid sequence of the DH*-KR* di-domain portion shows the highest similarity to a putative didomain protein (encoded by *orf19*) in a region spanning the biosynthetic gene clusters of lankacidin and lankamycin in *Streptomyces rochei* [38]. In the vicinity of *orf19*, two additional open reading frames, *orf21* and *orf22*, were identified (encoding an ACP and an FkbH homolog, respectively). Although disruptants of *orf19* seemed to be able to produce both lankacidin and lankamycin, the role of *orf21* and *orf22* in production of either antibiotic was not studied [38].

The lankacidin group of antibiotics contains a 3-carbon unit that assumes different oxidation/reduction levels in different antibiotic components, a pyruvate in lankacidin and a D-lactate in lankacidinol [39]. It is conceivable that the reaction scheme shown in Figure 5A, catalyzed by the enzymatic domains present in *orf22*, *orf21*, and *orf19*, could apply to the lankacidin group of antibiotics. Utilization of all four domains (FkbH, ACP, DH, and KR) would produce the 3-carbon unit found in lankacidinol, while skipping the last reaction (i.e., the α-ketoreduction) will leave pyruvate at the same position to form lankacidin. In this context, it is interesting to note that the consensus for the glycine-rich NADP(H) binding motif, GXXXGXGXXXAXXXA, is well conserved in the KR* in *bryA*, but less so in *orf19* from *S. rochei* (data not shown). Production of lankacidin in *orf19* mutants could be attributed to contribution from an unidentified redundant protein present in this mutant that compensates for the loss of the DH* activity. Alternatively, perhaps the construction of the mutant (which was not described) did not eliminate the DH* activity.

Another case in which the reaction scheme shown in Figure 5A may be functional is the biosynthesis of boron-containing antibiotic aplasmomycin produced by *Streptomyces griseus* [40]. Labeling studies with various substrates indicated that the biosynthetic precursor for the glycerol-derived starter unit of aplasmomycin is likely phosphoglycerate or phosphoenolpyruvate [40]. Interestingly, this 3-carbon starter unit also has a configuration of D-lactate. It is possible that our proposed reaction scheme involving FkbH, DH*, KR*, and ACP represents a common theme nature has developed for incorporation of a glycolytic pathway intermediate into polyketide biosynthesis.

The methyltransferase domain in *bryA* is unusual in a modular PKS, and in this case, its function, if any, is unknown, but it has been seen in other polyketide synthases, for example in the epothilone gene cluster in *Sorangium cellulosom* [32, 33]. In addition, the KRs of M1 and M2 are proposed to catalyze formation of hydroxyl groups with opposite stereochemistry (Figure 5B). Recent work by Caffrey [41] suggests that stereochemical specificity of KRs can be identified through characteristic sequence signatures. This study was done by using KRs from Streptomycete PKSs with integral ATs. Unfortunately, analysis of KRs from discrete AT PKSs does not support these patterns (data not shown). However, the last aspartate in the LDD signature associated with B-type KRs of integral AT PKSs does appear to be predominant in B-type KRs of discrete AT PKSs. The proposed B-type M2 KR contains this aspartate (Figure 3). This aspartate is generally absent in A-type KRs from discrete AT PKSs and is absent from the proposed A-type M1 KR.

BryA lacks integral ATs, as has been observed in several other modular PKS clusters, including the *pksX* of *B. subtilis*, TA antibiotic, mupirocin, pederin, and leinamycin clusters [2, 42–45]. Discrete ATs are known for some, but not all, of these. Recent work by Shen's group [45] characterized the leinamycin AT biochemically and showed that discrete ATs are evolutionarily distinct from integral ATs. These discrete ATs share a common evolutionary origin, despite the fact that this class of PKSs is not confined to any particular group of bacteria. Thus, there are two distinct classes of modular PKSs. One class, including the classical DEBS system, contains integral acyltransferases: a dedicated (integral) AT domain appears in each module. This allows further chemical diversity by permitting the incorporation of different extender units in each extension reaction; typically these are malonyl CoA or methylmalonyl CoA. The other class uses discrete ATs: one or two separate AT enzymes that serve multiple modules. Thus far, all of these are specific for malonyl CoA (the expected extender unit for bryostatin). The relationship of *bryA* KSs with those of other modular PKSs that use a separate, discrete AT (Figure 4) is consistent with a requirement for a discrete AT for *bryA* function. As noted by Piel [46], it also suggests that the discrete AT PKSs as a whole have distinct evolutionary origins. We are currently screening the symbiont genome for discrete AT domains.

BryA contains unusual and interesting features that will be worth exploring in future studies. The high conservation between shallow and deep sequences strengthens the hypotheses that the "*Candidatus* E. sertula" PKS cluster codes for enzymes involved in synthesis of the bryopyran precursor of the bryostatins and that the diversity of bryostatins is introduced by modifications after synthesis of the bryopyran ring. It is obvious that *bryA* functions as part of a much larger gene cluster in the biosynthesis of a complete bryostatin, and we are in the process of characterizing other genes in the "*Candidatus* E. sertula" PKS cluster.

Significance

Bryostatins are promising anticancer compounds, which are currently in clinical trials, but their availability is limited. The biosynthetic source of bryostatins is thought to be the bacterial symbiont, "*Candidatus* Endobugula sertula," of the marine bryozoan *Bugula*

***neritina*. "*Candidatus* E. sertula" is uncultivated, which has led us to explore unconventional means to obtain useful amounts of bryostatin. One approach is to clone genes involved in bryostatin synthesis for expression in a cultivatable host. We have isolated a large gene cluster from "*Candidatus* E. sertula" that encodes a modular polyketide synthase (PKS) complex, which we believe is responsible for bryostatin synthesis. We have characterized the first gene in this cluster, *bryA*, and present a model for its involvement in synthesizing a portion of bryostatin. We propose that *bryA* encodes the bryostatin loading module, including an FkbH homolog domain that is involved in an uncommon, but previously documented, series of reactions to synthesize the starter unit for bryostatin from a glycolytic pathway intermediate to form D-lactate. The proposed compound produced by the *bryA* gene product contains most of the pharmacologically active portion of bryostatin. Thus, expression of *bryA* in combination with a suitable, discrete AT domain could serve as the basis for the semisynthesis of a compound with bryostatin-like qualities. The isolation of *bryA* represents a significant step forward in understanding bryostatin biosynthesis and eventually harnessing *bry* genes to produce bryostatins and derivatives inexpensively and in abundant quantities.**

Experimental Procedures

Sample Collection and Genotype Determination

Samples of *Bugula neritina* were collected by SCUBA diving from three sites along the coast of San Diego, CA, USA; Mission Bay (water depth ~5 m, sample not genotyped, but previous collections determined to be type S), Scripps Pier (~7 m, type S), and Torrey Pines Artificial Reef II (~15 m, type D). The genotype of collected specimens was determined by amplifying the 16S gene of "*Candidatus* E. sertula" by PCR and digesting the product with the restriction enzyme NheI. Due to a sequence polymorphism, the deep type is cut into two fragments, whereas the shallow type is not [47].

DNA Isolation

Two methods were used for DNA isolation. The first, a "total" DNA preparation, was done by pulverizing 8 g aliquots of *Bugula neritina*, frozen at −80°C, into a fine powder in a dry-ice pre-chilled mortar and pestle. The powder was then added to 25 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM Na_2EDTA, 350 mM NaCl, 2% sodium sarcosyl, 8 M urea), mixed, and incubated at room temperature for 5 min. Ten ml phenol:chloroform (1:1) was added, and the solution very gently rotated (25 rpm) for 40 min to mix the layers. After centrifugation to separate the layers, the aqueous layer was removed with a wide-bore pipette into a new tube, and phenol:chloroform extracted for 20 min. The aqueous layer was removed, and one-tenth volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol were added and mixed. DNA immediately precipitated and was removed from the solution by spooling. Spooling was superior to centrifugation because it reduced the amount of a difficult-to-remove abundant pigment that co-pelleted with the DNA. The spooled DNA was washed with 70% ethanol, resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na_2EDTA), and then subjected to 53% cesium chloride (CsCl)/ethidium bromide equilibrium density gradient centrifugation. The resulting DNA band was collected, the ethidium bromide was removed by butanol extraction, and the CsCl was removed by dialysis.

The second DNA isolation method, "enriched bacterial fraction," incorporated two steps designed to enrich for "*Candidatus* E. sertula" DNA. Aliquots (33 g each, for a total of 133–166 g) of freshly harvested *B. neritina* was added to 100 ml chilled artificial sea water (ASW). Aliquots were homogenized for 1 min in a Polytron homogenizer (model PCV11) at setting 4. The slurry was distributed into tubes and centrifuged at 164 × g for 15 min to pellet cell debris. The supernatant was pipeted into a new tube and centrifuged at 16,500 × g for 15 min to pellet material of bacterial density. The pellet was resuspended in 5 ml lysis buffer and extracted as above. DNA was generally not visible upon precipitation, so instead of spooling, it was pelleted, washed, and then resuspended in TE buffer. Samples were then subjected to CsCl/ethidium bromide gradients as described above. After butanol extraction of ethidium bromide from the DNA, samples were run on 53% CsCl/Hoechst 33258 dye gradients (containing 10 μg/ml dye), which separates DNA on the basis of GC content. The region of the gradient containing the bands (at least four were visible) was fractionated by using a catheter attached to a peristaltic pump; for fine resolution, 70–140 μl drops were collected. Individual fractions were diluted 5-fold, and DNA was ethanol precipitated and resuspended in a small volume. Fractions shown to contain the KSa gene by PCR were pooled and used for subsequent cloning or analysis.

Competitor Construction and DNA Enrichment Assays

To quantify "*Candidatus* E. sertula" DNA content by competitive PCR, a competitor of KSa (clone pSWA116) was constructed by generating a 60 bp internal deletion to allow resolution from the authentic fragment on an agarose gel [1]. Competitive PCR [48, 49] was done with 200 ng of the DNA to be tested and different concentrations of the pSWA116 clone in the same reaction mix. KSa-specific primers were used to amplify products from native copies of the gene (assay DNA) and varying amounts of pSWA116 (competitor) simultaneously. PCR products were separated on an agarose gel, and conditions under which the full-length (assay DNA) and modified (pSWA116) products were equally abundant were determined. Under these conditions, the amount of KSa in the assay DNA and added pSWA116 were equal. Because the concentration of pSWA116 was known, the amount in the assay DNA could be calculated, allowing a comparison of enrichment of this gene fragment in the different samples tested [1].

Cloning, Sequencing, and Sequence Analysis

Cloning DNA from Mission Bay B. neritina *into Cosmid Vectors*

Total DNA of *B. neritina* from Mission Bay was partially digested in 2 μg aliquots by using a dilution series of Sau3AI to determine conditions yielding appropriate-sized (30–42 kbp) restriction fragments. Digests were then scaled up to 100 μg DNA, and products separated on 10%–40% sucrose gradients in a Beckman SW41 rotor, for 22 hr at 22,000 rpm and 20°C. The gradient was fractionated from the top by removing 350 μl aliquots with a pipetman. Portions of each aliquot were analyzed by agarose gel electrophoresis, and fractions containing DNA of the desired size were identified and pooled. DNA was ligated with BamHI-digested SuperCos I (Stratagene, Inc.) and packaged into phage (Gigapack Gold III, Stratagene, Inc.), which were titered according to the manufacturer's instructions. After initial characterization of selected cosmid clones, portions were subcloned into pBC+ (Stratagene, Inc.) by using standard procedures [50].

Cloning DNA from Scripps Pier B. neritina *into Lambda DASH II*

To isolate smaller portions of the putative bryostatin PKS cluster, as well as to obtain more complete coverage, we recloned the region into Lambda DASH II (Stratagene, Inc.), which accommodates fragments of 9–23 kbp. Aliquots (3 μg) of "enriched bacterial fraction" Scripps pier (type S) DNA were partially digested with Sau3AI, and one-third was electrophoresed on an agarose gel to assess the digestion. The remainder of digested DNA from conditions yielding appropriate-sized fragments was then phenol:chloroform extracted, precipitated, washed, and resuspended in TE buffer. DNA was electrophoresed on a 0.6% agarose gel, the region of the gel containing fragments between 9 and 23 kbp was excised, and DNA was extracted by using a Marligen Rapid Gel Extraction kit. Recovery of DNA was estimated by running an aliquot on an agarose gel. The appropriate amount of insert DNA was ligated with BamHI-digested Lambda DASH II (Stratagene) vector, packaged into phage, and titered, all according to the manufacturer's instructions.

Cloning DNA from Torrey Pines Artificial Reef B. neritina *into Lambda ZAP*

"*Candidatus* E. sertula"-enriched DNA isolated from Torrey Pines Artificial Reef (type D) was used to construct a Lambda ZAP (Stratagene, Inc.) library. Aliquots (2 μg) were partially digested with Sau3AI and electrophoresed on an agarose gel. The region between 4 and 6 kb was excised, and the DNA was isolated by using a Qiagen Gel Extraction kit. The DNA was then ligated with BamHI-digested Lambda ZAP vector and packaged into phage according to the manufacturer's instructions.

Library Screening and Clone DNA Isolation

For the cosmid library, approximately 14,000 colonies were plated and lifts were made by using established procedures [50]. Filters were screened with a radiolabeled probe derived from the KSa PCR product. Radiolabeling was done by using α-^{32}P dCTP and a Stratagene Prime-it II random priming kit. BLOTTO [42] in 2× SSC was used as a blocking agent for prehybridization (2 hr, 55°C), and probe was then directly added to 1×10^6 cpm per ml for hybridization at 55°C for 18 hr. Probe solution was removed, filters were rinsed twice (5 min for each rinse) with 2× SSC, 0.1% SDS at room temperature, and filters were then washed twice (45 min for each wash) with the same solution at 55°C. Filters were exposed to X-ray film overnight, which was then developed. A similar procedure was used with the Lambda Dash and Lambda ZAP libraries, in which approximately 40,000 and 50,000 plaques were screened, respectively. Probes for screening the Lambda libraries were derived from cosmid fragments.

DNA was isolated from cosmid clones by using a Qiagen miniprep procedure. DNA was isolated from lambda phage (DASH library) by established procedures [50]. Plasmid rescue for Lambda ZAP clones was done according to the manufacturer's instructions; DNA was isolated from these plasmids by minipreps.

Completion of Deep bryA *by PCR*

Due to a lack of Sau3AI sites, the first third of *bryA* was not present in the Lambda ZAP library. This part of the gene had to be amplified from total *B. neritina* DNA and TOPO-TA cloned (Invitrogen). The primers were designed by using sequence information from the shallow genotype. Possibly due to the extremely low GC content in this part of the gene (~20%), amplification of more than ~1 kb was not achievable, but four overlapping fragments were generated. The PCR reactions were run by using the Expand Long Template PCR system (Roche) following manufacturer's instructions.

Sequencing and Sequence Analysis

DNA from isolated clones was sequenced (2- to 3-fold coverage, both strands) on ABI 3100 automated DNA sequencers at the UCSD sequencing facilities in the Rebecca and John Moores UCSD Cancer Center and Veterans Medical Research Foundation/UCSD Center for AIDS Research with vector-specific primers. Sequential sets of primers to extend the sequences were designed based on accumulating sequence data.

Sequence reads were assembled and analyzed with Sequencher (Gene Codes Corp.). Repeats were identified by dot plot analysis in Vector NTI (Informax Inc.). Domains within the sequenced clones were identified by homology with NCBI BLAST [30] and through manual alignment with other known PKS domains.

We also analyzed the relationship between KS domains by aligning the amino acid sequence of the BryA domains with those from other modular Type I PKS gene clusters, as well as a Type II PKS KS domain, by using CLUSTALW [51]. We performed phylogenetic analysis with PAUP [52] and used distance as the optimality criterion, heuristic search settings, and random addition of taxa. The resulting tree was bootstrapped by using 1000 replicates.

Restriction Mapping and Southern Blotting

Southern blot hybridization analysis was performed on genomic DNA to confirm restriction maps determined by subcloning and sequencing of the *bryA* PKS region. 3 μg genomic "*Candidatus* E. sertula"-enriched DNA and 30 ng cloned DNA were digested with 10 U of appropriate restriction enzymes for 3 hr at 37°C in a universal buffer (20 mM Tris [pH 7.5], 70 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 50 μM spermine tetrahydrochloride, 12.5 μM spermidine trihydrochloride, and 10 μM DTT). An additional 10 U of each enzyme was then added, and the reaction proceeded for 1 hr. Restriction enzymes were chosen based on their ability to cut the DNA into appropriately sized fragments for use in Southern hybridizations. The following single and double digests were performed: BamHI, PstI, PstI/XhoI, SmaI, SmaI/NotI, XhoI/SalI, and SalI/EcoRI. The resulting fragments were electrophoresed on a 0.8% agarose gel and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech) by using standard protocols [50]. Based on the cloned sequence, probes were designed to cover the entire *bryA* region. These were generated by PCR amplification, and radiolabeling was done with α-^{32}P dCTP and a Stratagene Prime-it II random priming kit. Southern hybridizations were performed according to standard protocols [50], and localization of radioactive bands was visualized with a Typhoon 9410 Variable Mode Imager (Molecular Dynamics) after an 18 hr exposure. Genomic and corresponding cloned DNA fragments were compared by size.

Table 2. Primers for RT-PCR in *bryA*

RT-PCR Region	Primer Name	Sequence 5′-3′
A	BryA-DHLF	GGAAGAAGTTGATTGGTTTTCA
	BryA-DHLR	CTCAACACCTTTACTCGCTATC
B	BryA-ACPLF	CGCTAAAGTATTAGCGCGACGC
	BryA-ACPLR	ATTCGCTCTTTTTGATGCCTGC
C	BryA-KR1F	CGCTCGTAAACTTGCAAACATG
	BryA-KR1R	TGTGTTGAATTCCAGCCGCATG
D	BryA-MT3F	GAACGTTTTGCTGCGATCCA
	BryA-MT3R	GTCTCTTCTACATGAAAACC

RNA Isolation and Reverse Transcription PCR

Two 7.5 g aliquots of Scripps Pier (type S) adult *B. neritina* were homogenized in 20 ml artificial sea water with a Polytron homogenizer. An enriched bacterial fraction was prepared as described for DNA isolation. The cell pellet was resuspended in 3.1 ml Tri Reagent (Sigma). RNA was isolated following the manufacturer's instructions and resuspended in 500 μl RNase-free water (Qiagen). In order to remove PCR inhibitors, the RNA was purified by using MicroSpin S-400 HR columns (Amersham Biosciences) with 50 μl RNA solution per column. To remove contaminating DNA, purified RNA was then treated with DNase I (Qiagen) on an RNeasy Mini Kit spin column (Qiagen), as described by the manufacturer, and eluted with 30 μl RNase-free water (Qiagen).

cDNA was synthesized from 7 μl DNase-treated RNA per reaction by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Each reverse transcription reaction contained 1 μl of a 2 μM gene-specific primer, BryA-DHLR, BryA-ACPLR, BryA-KR1R, or BryA-MT3R (Table 2). A total of 10 μl of the cDNA product was added to a 50 μl PCR reaction containing 1 μM of each primer in the corresponding primer pair (Region A–D, Table 2), 1.25 U Taq DNA polymerase (Roche), 1× PCR reaction buffer (Roche), 0.5 ng BSA, and 0.2 mM dNTPs (Invitrogen). PCR reactions were done under the following conditions: one cycle of 1.5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C; and 1 cycle of 7 min at 72°C. RT-PCR products were run on a 1.2% agarose gel and sequenced. To confirm that RT-PCR products reflected RNA content rather than DNA contamination, control RT-PCRs without reverse transcriptase were conducted for each primer pair.

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Accession Numbers

The shallow and deep *bryA* sequences have been submitted to GenBank with accession numbers AY553931 and AY553932, respectively.